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Role of pituitary tumour transforming gene 1 in medullary thyroid carcinoma

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Abstract. *Background*: Pituitary tumour transforming gene 1 (*PTTG1*) is over-expressed in a variety of endocrine-related tumours. We aimed at evaluating *PTTG1* expression and function in human neoplastic parafollicular C-cells, represented by medullary thyroid carcinoma (MTC) and C-cell hyperplasia (CCH) samples and by the TT cell line.

Methods: TT cells and tissues derived from human CCH (8 samples) and MTC (12 samples) were analyzed by northern blot, furthermore TT cells were subjected to *PTTG* gene silencing and cells were analyzed for DNA synthesis.

Results: *PTTG1* expression was significantly higher ($p < 0.01$) in CCH (3-fold), in papillary thyroid cancer and in MTC (5-fold) than in normal thyroid, and in MTC lymph-node metastases as compared to primary lesions ($\sim$2-fold; $p < 0.05$). *PTTG1* mRNA and protein correlated with tumour diameter and TNM status ($p < 0.05$). In TT cells, *PTTG1* silencing did not completely block DNA synthesis, but significantly reduced [^{3}H]Thymidine incorporation ($\sim$50%; $p < 0.01$) for up to 3 days.

Conclusions: *PTTG1* levels correlate with tumour aggressiveness. *PTTG1* silencing causes reduced MTC cell proliferation, supporting the hypothesis that *PTTG1* might have an important role in C-cell neoplastic proliferation.

Keywords: Pituitary tumour transforming gene 1, medullary thyroid carcinoma, TT cells, siRNA

1. Introduction

Pituitary tumour transforming gene 1 (*PTTG1*) is over-expressed in a variety of endocrine-related tumours, as well as non-endocrine cancers [15], where high *PTTG1* expression correlates with a poor prognosis [22,30]. *PTTG1* is a multifunctional human securin, involved in the control of mitosis [23,31], cell transformation [8,29], DNA repair [16] and gene regulation [13,14,29]. *PTTG1* also exhibits transactivational activity [21]. *PTTG1* expression is higher in recurrent as compared to primary differentiated thyroid cancer [3], supporting the hypothesis that *PTTG1* is important for thyroid cancer progression. On the other hand, *PTTG1* overexpression in mouse NIH 3T3 fibroblasts inhibits cell proliferation and induces cell transformation *in vitro* [15]. It has indeed been previously demonstrated that the multifunctional *PTTG1*-encoded protein, securin, markedly influences cell proliferation depending on its expression levels, suggesting that altered *PTTG1* expression may differentially affect cell turnover. In fact, low levels of *PTTG1* expression stimulate cell proliferation, while at higher levels it inhibits cell turnover [4].

Involvement of *PTTG1* in medullary thyroid carcinoma (MTC) has not thus far been tested. MTC, derived from parafollicular thyroid C-cells, accounts for 3–5% of cases of thyroid cancer [5,18] and is characterized by early metastases and poor prognosis. Understanding molecular pathways involved in the control of MTC and C-cell hyperplasia (CCH) development is fundamental for applying novel therapies for patients with advanced MTC. Indeed, the majority of MTC patients have persistent or recurrent post-operative disease which is not amenable to medical therapy.

In the present study we investigated *PTTG1* expression in human CCH, MTC and in the human MTC cell

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line, TT. Moreover, the role of *PTTG1* in neoplastic C-cell proliferation was studied by modulating *PTTG1* expression.

2. Material and methods

2.1. Patients

Samples were derived from 8 patients operated on for CCH, 12 patients operated on for primary MTC, and 7 patients operated on for MTC neck lymph-node metastases, as confirmed by histology and immunohistochemistry. Tissues were harvested under sterile conditions, as previously described [28]. Surgical procedures were performed at the Section of General Surgery of the University of Ferrara and at the Department of General Surgery of the University of Padova. Tissues derived from papillary thyroid carcinoma (PTC) samples were considered as positive controls. Tissues were microdissected as previously described [11]. Table 1 shows individual patient characteristics, including demographic details, tumour node metastasis (TNM), staging at surgery, Calcitonin (CALC) plasma levels and tumour size. Tumour size of 2 MTC patients was not available (#22 and #24). All patients (12 males and 15 females; aged 52.6 $\pm$ 4.2 years) underwent total thyroidectomy with central neck lymph-node clearance. Patient informed consent was obtained for disclosing clinical investigation and performing the *in vitro* study that was approved by the local Ethical Committee.

Table 1
Characteristics of CCH and MTC patients

No.	Age	Sex	Inheritance	TNM	STAGE	Plasma CALC (pg/ml)	Lesion type	Max diameter (cm)
1	60	F	SP	–	–	332	P	–
2	10	M	MEN 2A	–	–	59	P	–
3	11	F	MEN 2A	–	–	84	P	–
4	51	M	SP	–	–	93	P	–
5	51	M	SP	–	–	157	P	–
6	35	M	SP	–	–	68	P	–
7	62	F	SP	–	–	136	P	–
8	12	M	MEN 2A	–	–	73	P	–
9	69	F	SP	T2NxMx	I	410	P	0.7
10	76	F	SP	T1N0Mx	II	1500	P	3.9
11	56	M	SP	T1NxMx	I	875	P	0.6
12	69	F	SP	T2N1Mx	III	1950	P	2.2
13	73	F	SP	T1N0Mx	I	163	P	0.8
14	18	F	MEN 2A	T1N0Mx	I	69	P	1.8
15	77	F	SP	T1N0Mx	I	217	P	3.4
16	61	M	SP	T3N0Mx	II	2580	P	4.5
17	38	F	SP	T2N0Mx	II	2350	P	4.0
18	53	F	SP	T2N0Mx	II	306	P	1.6
19	63	F	SP	T2N0Mx	II	74.5	P	1.6
20	27	F	MEN 2A	T2NxMx	II	940	P	2.0
21	66	M	MEN 2A	TxN1Mx	III	116.5	L	1.0
22	86	F	SP	TxN1Mx	III	776	L	n.d.
23	67	M	SP	TxN1Mx	III	1530	L	0.7
24	71	F	SP	TxN1Mx	IV	140	L	n.d.
25	66	M	SP	TxN1Mx	III	1500	L	2.0
26	52	M	SP	TxN1Mx	III	1831	L	1.4
27	42	M	SP	TxN1Mx	IV	9227	L	4.3

Notes: Demographic details (age and sex), inheritance (SP: sporadic; MEN2A: patients with MEN2A RET mutation), TNM classification, staging, plasma CALC levels, lesion type (P – primary, L – lymph node metastases) and maximal tumour diameter. Patients #1–8 suffered from CCH; patients #9–27 suffered from MTC.

2.2. Materials

All reagents, if not otherwise specified, were purchased from Sigma-Aldrich (Milano, Italy).

2.3. TT cell line

The TT cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in culture in F-12 Ham's medium (F-12) and 10% fetal bovine serum (FBS), as described [24]. TT cells express and secrete CALC, carcinoembryonic antigen, chromogranin A, and other peptides. TT cells harbour a MEN 2A-type mutation, with a cysteine-to-tryptophan substitution at the level of RET codon 634, and a RET polymorphism glycine-to-serine at codon 691 in exon 11.

2.4. RNA isolation

Total RNA was isolated from pulverized tissues and from subconfluent TT cells by Trizol reagent (Invitrogen, Milano, Italy), according to the manufacturer's protocol. To prevent DNA contamination, RNA was treated with RNAse-free deoxyribonuclease (Promega, Milano, Italy). RNA quality and quantity were assessed with the Experion automated electrophoresis system using the Experion RNA StdSens analysis kit (Bio-Rad, Hercules, CA, USA).

2.5. Qualitative RT-PCR

Reverse transcription reaction was performed as previously described [28]. Briefly, cDNA (1 μl of RT reaction) was amplified by PCR with 1 U Taq DNA polymerase (Invitrogen). PCR reactions for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *CALC* were carried out as previously described [26]. PCR primers for Thyroglobulin were as follows: forward 5′-TGGATGGAAACAACCCTAGCT-3′; reverse 5′-TCCCACGTAAGATGTAACCCA-3′. PCR primers for *PTTG1* were as follows: forward 5′-CCCGTGTGG TTGCTAAGGAT-3′; reverse 5′-GTCACAGCAAA CAGGTGGCA-3′. Both amplifications were carried out for 40 cycles with the following conditions: denaturation for 30 s at 95°C; annealing for 1 min at 65°C and 45 s at 57°C; extension for 2 min at 72°C and for 45 s at 74°C, respectively.

2.6. Northern blotting

To evaluate *PTTG1* gene expression in CCH, MTC, PTC and normal thyroid samples, Northern blot analysis was performed as described [26]. Briefly, probes were obtained by labeling fragments derived from *CALC* (UniProtKB database Accession P01258) *PTTG1* and *GAPDH*. Results are expressed as the ratio between *PTTG1* and *GAPDH* signal intensities. Experiments were performed with at least 3 replicates and results expressed as mean ± SE.

2.7. Protein isolation and Western blot analysis

PTTG Western blot analyses were performed as described previously [6,20]. Membranes were blotted overnight with anti-PTTG1 antibody (2 μg/ml) (sc-56207; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with GAPDH antibody (1 μg/ml). Quantification of bands was carried out using the Quantity-One software (Bio-Rad). Data are expressed as the ratio between PTTG1 and GAPDH signal intensity. Experiments were performed with 3 replicates and results expressed as mean ± SE.

2.8. PTTG1 silencing

For gene silencing, 3 different anti-*PTTG1* (gene bank accession no. NM_004219) siRNAs, pre-designed and synthesized by Ambion (St. Austin, TX, USA) were transfected into TT cells by nucleofection. The ID number and targeted sequences are as follows: 289470 (*PTTG1* siRNA #1) – Sense 5′-UCCAAUCUGUUGC AGUCUCtt-3′, Antisense: 5′-GAGACUGCAACAGA UUGGAtt-3′; 41990 (*PTTG1* siRNA #2) – Sense 5′-GUCUGUAAAGACCAAGGGAtt-3′, Antisense: 5′-UCCCUUGGUCUUUACAGACtt-3′; 42068 (*PTTG1* siRNA #3) – Sense 5′-GAGUUUGUGUGUGUAUU UGUAtt-3′, Antisense: 5′- UACAAAUACACACAAA CUCtg -3′. Positive controls were employed for gene specificity and for optimizing siRNA experiment (*GAPDH* siRNA; Ambion, ID: AM4624). Non-targeting control siRNA (Scramble siRNA; Ambion, ID: AM4644) was used for non-specific gene silencing. For evaluation and optimization of transfection efficiency 1.5 μg pmaxGFP plasmid (Amaxa, Cologne, Germany) was used for each nucleofection, performed using the cell line optimization Nucleofector Kit (Amaxa) following the manufacturer's protocol. The same conditions were used for siRNA transfections, by using, in total, 100 nM siRNA in TT cells serum-

starved for 48 h. After optimized nucleofection, cells were immediately transferred to six-well plates, in pre-warmed RPMI medium with 10% FBS, and incubated for at least 24 h, prior to further experiments. Cells, total RNA, and cell lysates were collected for immunofluorescence, real-time PCR and Western blot studies to determine *PTTG1* expression levels.

2.9. Quantitative PCR for human PTTG

To evaluate *PTTG1* silencing by siRNAs in TT cells, relative quantitative PCR (RQPCR) and Comparative C_T Method (User Bulletin no. 2, Applied Biosystems) with the predeveloped Taqman Assay Reagent (no. 4331182) were performed by using Assay on Demand Hs00851754_u1 (Applied Biosystems) and huPO as reference gene, as previously described [27,28].

To demonstrate specificity of our gene silencing strategy, we also evaluated CALC gene expression by RQPCR, as described above, by using Assay on Demand Hs00266142_m1 (Applied Biosystems).

RQPCR reactions were performed, recorded and analysed using the ABI 7700 Prism Sequence Detection System with the SDS software 1.9 (Applied Biosystems). Samples were carried out in triplicate (50 ng reverse transcribed total RNA per well) and repeated at least twice. Controls without template or RT were run in each experiment.

2.10. Fluorescence microscopy

To evaluate securin expression in control, *PTTG1* silenced TT cells, 2×10^4 cells/well were seeded in 8-well chamber slides (Lab-Tek Chamber Slide System, Nalgene Nunc International, Naperville, IL, USA) and incubated 30 min in a humified atmosphere at 37°C with a mouse monoclonal anti human PTTG1 antibody (3.5 μg/ml) (Zymed Laboratories). Cells were fixed in methanol-acetone (1:1) for 10 min at −20°C, blocked for 1 h with blocking buffer (5% goat serum in PBS), and incubated with a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti mouse antibody (1:200; Invitrogen Molecular Probes, Eugene, OR, USA) for 45 min at room temperature. Slides were mounted with the ProLong Gold antifade reagent (Invitrogen Molecular Probes) containing the nuclear stain 4′,6′-diamidino-2-phenylindole (DAPI) under glass coverslips (Menzel-Glaser, Braunschweig, Germany). Slides were visualized with a Nikon Eclipse TE2000-U fluorescent microscope, photographed with a ×20 or ×60 objective magnification with a DS-5M Nikon colour CCD digital camera and analyzed with the Multi-Analyst software (Bio-Rad). Pre-immune serum and antigen-absorbed antibody were used as controls. All experiments were carried out independently at least three times and 50 ± 10 individual cells analysed.

2.11. DNA synthesis

To investigate the effects of *PTTG1* silencing on DNA synthesis, [^{3}H]Thymidine ([^{3}H]Thy) incorporation was measured in control, and in *PTTG1* silenced cells, as described [25]. Cells were incubated in Ham's F-12 medium without serum for 36 h, and then cultured in Ham's F-12 medium supplemented with 10% FBS for up to 72 h in the presence of [^{3}H]Thy (1.5 μCi/ml; 87 Ci/mmol, Amersham–Pharmacia Biotech Italia, Cologno Monzese, Italy) for the last 24 h before harvesting the cells. After incubation, cell-associated radioactivity was determined after harvesting cells on glass fibers, and liquid scintillation counting in at least three separate experiments. Results are calculated as average counts per minute of quadruplicate wells ± SE.

2.12. Statistical analysis

Data are expressed as mean ± SE. A preliminary analysis was carried out to determine whether the data sets conformed to a normal distribution, and a computation of homogeneity of variance was performed using Bartlett's test. Results were compared within each group and between groups using ANOVA. If F values were significant ($p < 0.05$), Student's paired or unpaired t-test was used to evaluate individual differences between means. To measure the strength of association between pairs of variables without specifying dependencies, Spearman order correlations were run. $p < 0.05$ was considered significant in all tests.

3. Results

3.1. PTTG1 mRNA expression in CCH, MTC, PTC and normal thyroid tissue

Northern blot analysis showed that *PTTG1* mRNA expression was significantly higher in CCH (3-fold), in PTC and in MTC samples (5-fold) than in normal thyroid (NT) specimens (Fig. 1; $p < 0.01$). *PTTG1* mRNA expression was higher in MTC as compared to

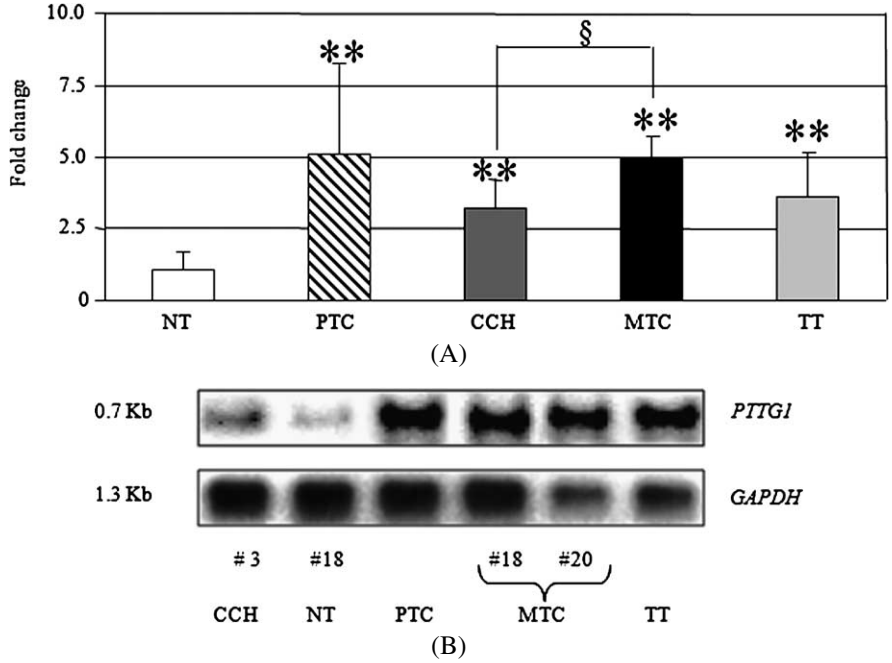

Fig. 1. Northern blot analysis of *PTTG1* gene expression. (A) Northern blot analysis for *PTTG1* mRNA expression in normal thyroid tissue (NT), 4 PTC, 8 CCH, 19 MTC and TT cells, expressed as fold changes vs. NT ± SE. $^{**}p < 0.01$, *PTTG1* mRNA expression vs. NT; $^{\S}p < 0.05$, *PTTG1* mRNA expression in CCH vs. MTC. NT – normal thyroid; PTC – papillary thyroid carcinoma; CCH – C-cell hyperplasia; MTC – medullary thyroid carcinoma; TT – TT cell line. (B) *PTTG1* mRNA levels. Representative Northern blot analysis for *PTTG1* and *GAPDH* expression in C-cell hyperplasia (CCH), normal thyroid (NT), papillary thyroid carcinoma (PTC), medullary thyroid carcinoma (MTC) samples and TT cells. Depicted results are representative of at least 3 different experiments.

CCH ($p < 0.05$), and 2-fold higher in MTC lymph-node metastases as compared to primary thyroid lesions (Fig. 2A; $p < 0.05$). Regression analysis showed a positive correlation between TNM status and *PTTG1* mRNA expression in CCH and MTC samples ($p < 0.05$). Similarly, *PTTG1* mRNA expression in 12 MTC samples derived from primary thyroid lesions correlated with tumour diameter ($r^2 = 0.693$; $p < 0.01$) (Fig. 2B).

3.2. PTTG1 protein levels in CCH and MTC

Western blot analysis of CCH and MTC protein extracts confirmed the mRNA results, since *PTTG1* protein expression was greater in tumour samples as compared to normal thyroid tissue, and in MTC as compared to CCH (Fig. 2C). Regression analysis evaluating tumour diameter and *PTTG1* protein levels in MTC samples demonstrated a correlation between these two parameters ($r^2 = 0.7981$; $p < 0.001$) (Fig. 2D).

3.3. Evaluation of PTTG1 gene silencing

For *PTTG1* silencing, we used three different siRNA, derived from various regions of the *PTTG1* coding sequence; a transfection efficiency of about 80% was achieved (data not shown). The extent of *PTTG1* gene silencing was evaluated by RQPCR in TT cells 72 h after transfection. A substantial and consistent reduction in *PTTG1* mRNA levels was observed after transfection with *PTTG1* siRNAs #1 and #3 as compared to untransfected control cells (18% and 36% PTTG expression relative to control cells, respectively; $p < 0.01$) (Fig. 3A). siRNA transfection determined a specific *PTTG1* mRNA suppression as shown by a non significant reduction in *PTTG1* expression in TT cells transfected with *GAPDH* siRNA or with scramble siRNA. Further confirmation of gene silencing specificity was provided by the evidence that *GAPDH* mRNA was significantly reduced in TT cells transfected with *GAPDH* siRNA but not in *PTTG1* silenced cells (data not shown). We therefore selected *PTTG1* siRNA #1 and #3 for further experiments. To further confirm the specificity of our gene silencing approach, *CALC* mRNA levels were measured by RQPCR in control untransfected TT cells, as well as in TT cells transfected with scramble siRNA, *GAPDH* siRNA, or with *PTTG1* siRNAs #1 or #3.

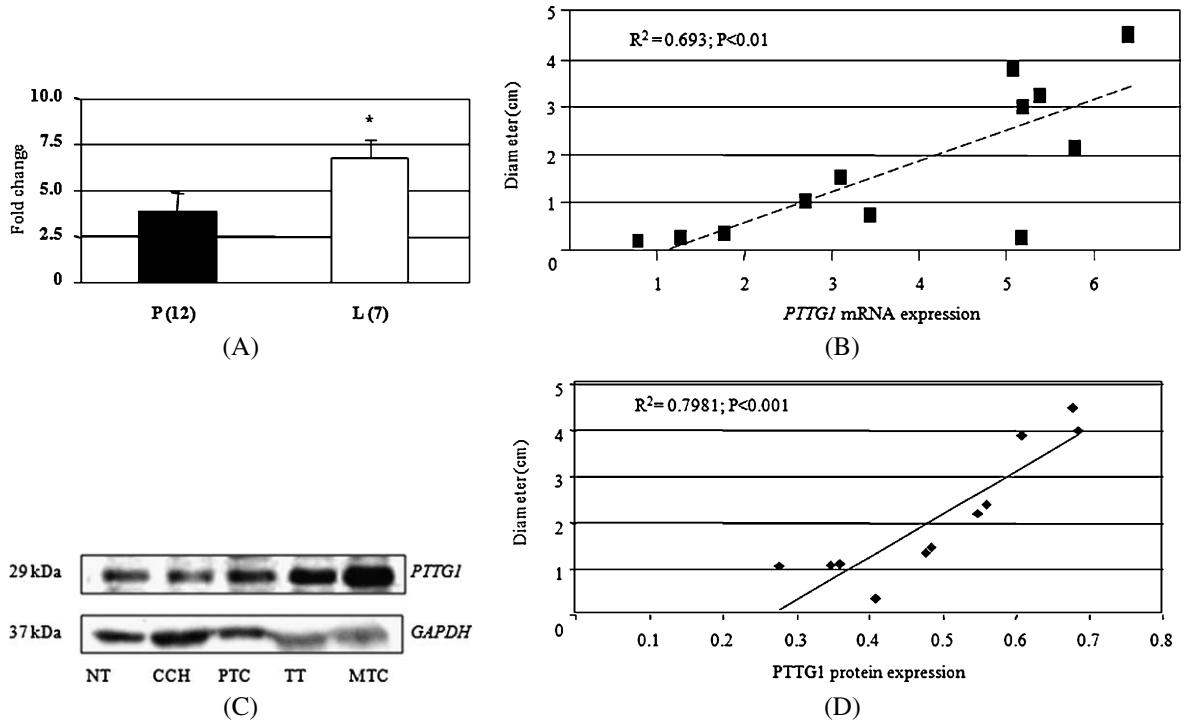

Fig. 2. *PTTG1* mRNA and protein expression in human MTC. (A) Mean *PTTG1* mRNA expression in 12 MTC primary thyroid lesions (P) and in 7 MTC lymph node metastases (L) expressed as fold change vs. normal thyroid tissue ± SE. $^{*}p < 0.05$ *PTTG1* mRNA expression in L vs. P. (B) Correlation between MTC tumour diameter (cm) and *PTTG1* mRNA expression (Arbitrary units). (C) PTTG1 protein levels. Representative Western blot analysis for PTTG1 and GAPDH protein expression in normal thyroid (NT), C-cell hyperplasia (CCH), papillary thyroid carcinoma (PTC), TT cells and in medullary thyroid carcinoma (MTC) samples. Whole cell extracts (80 μg) from each sample were fractionated on 12.5% SDS-PAGE, transferred on nitrocellulose membrane and incubated with an anti-PTTG1 antibody and, after stripping, with an anti-GAPDH antibody. (D) Correlation between MTC tumour diameter (cm) and PTTG1 protein levels (Arbitrary units).

Figure 3B shows that *CALC* mRNA levels were comparable in all tested samples, indicating that *PTTG1* gene silencing did not influence expression of other genes.

To evaluate whether reduced *PTTG1* mRNA after *PTTG1* gene silencing corresponded to decreased *PTTG1* protein levels, we performed immunofluorescence studies in control untransfected TT cells and in TT cells transfected with scramble siRNA, *GAPDH* siRNA, or with *PTTG1* siRNAs #1 and #3. Seventy two hours after transfection, cells were incubated with a specific *PTTG1* antibody, fixed, incubated with secondary FITC-conjugated antibody, and observed for specific immunofluorescence. As shown in Fig. 4, *PTTG1* immunofluorescence was clearly visible in control untransfected TT cells. Transfection with scramble siRNA did not affect *PTTG1* protein expression, while in cells transfected with *PTTG1* siRNA #1 or #3 specific *PTTG1* immunofluorescence was greatly reduced.

3.4. *Effect of PTTG1 silencing on [^{3}H]Thy incorporation in TT cells*

To test the effects of *PTTG1* silencing on TT cell proliferation, we evaluated DNA synthesis by measuring [^{3}H]Thy incorporation for up to 72 h after gene silencing. Figure 5A shows that transfection with *PTTG1* siRNA #1 and #3 resulted in reduced [^{3}H]Thy incorporation at 72 h, as compared to TT cells transfected with GAPDH siRNA (-47% and -51%, respectively; $p < 0.01$). Transfected cells continued incorporating [^{3}H]Thy, indicating that DNA synthesis was not completely blocked. DNA synthesis in TT cells transfected with scramble siRNA or with *GAPDH* siRNA was similar to that observed in untransfected control TT cells. These results indicate that the observed effects on cell proliferation depend on specific gene silencing (i.e., *PTTG1* down-regulation) and not on the transfection procedure per se.

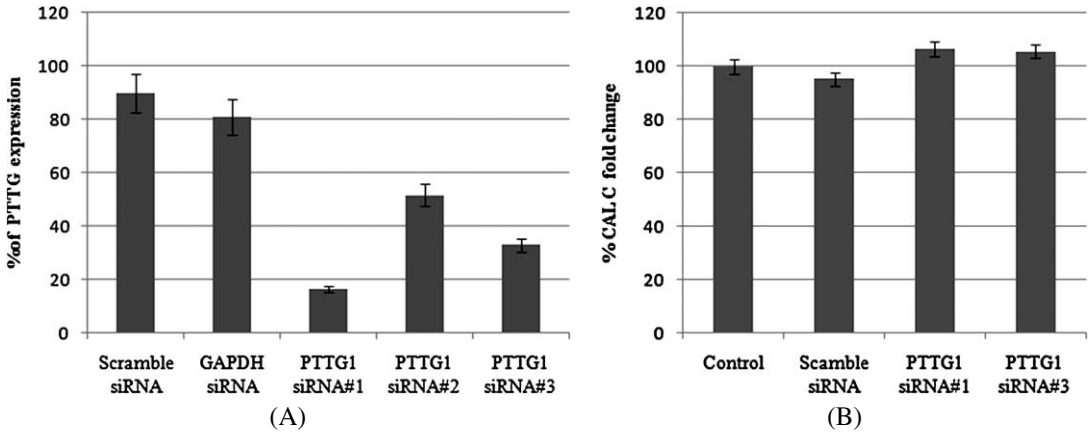

Fig. 3. RQ-PCR evaluating the effect of gene silencing on *PTTG1* and *CALC* mRNA expression in TT cells. (A) TT cells were transfected with scramble siRNA, *GAPDH* siRNA, or with *PTTG1* siRNA #1, #2 and #3. After 72 h, cells were harvested and used for RNA isolation. One μg RNA was reverse-transcribed and RQ-PCR performed to evaluate *PTTG1* mRNA expression. Data are shown as percentage of *PTTG1* expression in transfected TT cells as compared to control untransfected TT cells (100%), and represent the mean value ± SE of at least three independent experiments. $^{**}p < 0.01$ vs. control untransfected TT cells. (B) TT cells were transfected with scramble siRNA, *GAPDH* siRNA, or with *PTTG1* siRNA #1 and #3. After 72 h, cells were harvested and submitted to RNA isolation. One μg of RNA was reverse-transcribed and RQ-PCR performed to evaluate *CALC* mRNA expression. Data are shown as percentage vs. *CALC* expression in TT cells transfected with *GAPDH* siRNA, *PTTG1* siRNA #1–3 as compared to untransfected TT cells and represent the mean value ± SE of at least three independent experiments.

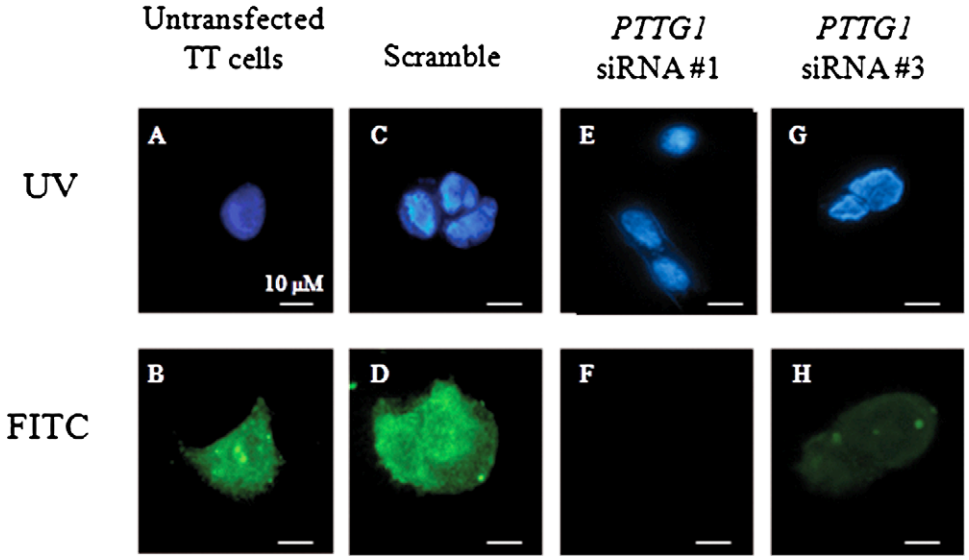

Fig. 4. *PTTG1* protein expression. TT cells were transfected with scramble siRNA, or with *PTTG1* siRNA #1 and #3 and fixed 48 h after transfection. Untransfected and transfected TT cells were incubated with the specific *PTTG1* antibody, fixed, incubated with the secondary FITC-conjugated antibody, mounted with the antifade reagent containing DAPI, and photographed with a 60× objective magnification. Cells were observed with the FITC filter, revealing specific PTTG1 immunofluorescence, and with the UV filter, revealing nuclear fluorescence. Figures are representative of at least three experiments.

4. Discussion

Human *PTTG1*, originally isolated from rat prolactin-secreting cells and encoded by a gene located on chromosome 5 [22], play a role in tumour initiation and progression [29]. *PTTG1* mRNA levels are increased in human thyroid tumours, compared with normal thyroid tissue. Increased *PTTG1* expression was detected early in thyroid tumour development, progressively increasing in thyroid hyperplasia, follicular ade-

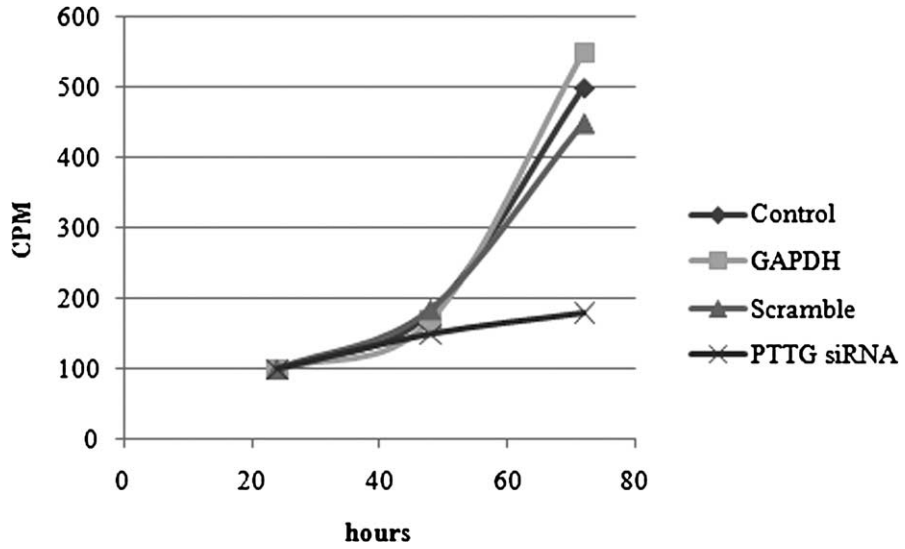

Fig. 5. Effect of *PTTG1* silencing on [^{3}H]Thy incorporation in TT cells. Panel A: TT cells were transfected with *PTTG1* siRNA #1 and #3, positive control *GAPDH* siRNA or negative control scramble siRNA, and cultured for up to 72 h. DNA synthesis was evaluated by measuring [^{3}H]Thy incorporation (cpm) in at least 3 independent experiments in transfected and in control untransfected cells (Control) after 48 and 72 h. Results were calculated as average counts per minute of quadruplicate wells ± SE.

nomas, papillary cancer and follicular carcinomas [7]. *PTTG1* overexpression was associated with the presence of nodal or distant metastases, TNM stage and decreased radioiodine uptake during follow-up, suggesting that *PTTG1* expression might represent an independent prognostic factor for persistent disease in the settings of papillary and follicular thyroid cancer [3]. However, to our knowledge, this is the first study investigating the role of *PTTG1* expression in human MTC. To study the role of *PTTG1* in parafollicular C-cell neoplastic transformation, we explored the hypothesis that *PTTG1* expression in human CCH and MTC is a useful marker of cancer behavior. Indeed, we found that *PTTG1* expression increases with disease progression, as also underscored by the correlation with primary lesion diameter and with TNM stage at presentation. Moreover, *PTTG1* expression was higher in human CCH and MTC as compared to normal thyroid tissue. This finding might also be due to the fact that CCH and MTC are composed by C-cells, while normal thyroid samples are mainly comprised of follicular thyroid cells. Evaluation of normal C-cell gene expression is hampered by the fact that they represent only a minority of the normal thyroid gland cell population. On the other hand, we found most abundant *PTTG1* expression in MTC as compared to CCH, which represents a widely accepted pre-malignant step in C-cell neoplastic transformation [12]. These results are in accordance with the putative role of *PTTG1* in C-cell neoplastic modification, suggesting that *PTTG1* overexpression associates with disease progression, as demonstrated in other endocrine tumours [19]. However, since the number of examined samples is low due to the rarity of the disease, to clarify this issue further evidence was sought in a cell line model.

To verify whether *PTTG1* influences C-cell neoplastic proliferation, we explored the effects of *PTTG1* gene silencing in human MTC cells, the TT cell line. We demonstrate that TT cells are a reliable model to investigate *PTTG1* activity, since this cell line expresses the gene at high levels, similar to those found in human MTC.

Our results show that gene silencing effectively down regulates *PTTG1* expression, without influencing the expression of other C-cell specific genes, such as CALC. Therefore, modifications in TT cell parameters after *PTTG1* silencing appear to be specific.

Our results show that specific *PTTG1* silencing induces a significant reduction in DNA synthesis, and reduced cell proliferation rates persist for at least 3 days, indicating that *PTTG1* expression down-regulation slows TT cell proliferation. This evidence supports the hypothesis that *PTTG1* plays an important role in C-cell neoplastic proliferation, suggesting a proliferative requirement for this gene in MTC development. Indeed, when considered as an oncogenic protein, *PTTG1* is expected to have pro-proliferative effects, as also indicated by others [9]. On the other hand,

as a securin protein which normally inhibits cell division, high *PTTG1* expression levels would be expected to inhibit cell proliferation, as demonstrated by Yu et al. [23]. It has been previously suggested that the stimulatory/inhibitory role of PTTG upon cell proliferation may be regulated by PTTG1 phosphorylation status, by *PTTG1* specific ability to degrade exogenous *PTTG1*, or may be dose-dependent [17]. Moreover, it has been previously demonstrated that transfection of FTC133 cells with PTTG1 dose-dependently increased genetic instability [10]. A high degree of genetic instability is also related to a reduced tumour growth potential, since it targets tumour cells to death [2]. Our results are in line with the hypothesis that PTTG acts as an oncogene in C-cells.

In conclusion, our work provides evidence for an important role of *PTTG1* in MTC cell proliferation.

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